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(54) PROCESS FOR PREPARING 5'-GUANYLIC ACID.

(57) GMP can be prepared in a good yield by converting XMP, ammonia and/or L-glutamine in an aqueous solution using a culture product, cells or their treated product of E. coli having GMP synthetase activity and the ability of converting AMP to ATP in the presence of an energy source other than phosphorus oxides, in the presence of the energy source. GMP can also be prepared in a good yield by converting XMP, ammonia and/or L-glutamine to GMP in an aqueous medium in the presence of ATP using a culture product, cells or their treated product of transformant obtained by using a recombinant DNA of a DNA fragment containing GMP synthetase gene and a vector DNA fragment. The process of using transformant having ATP-reproducing ability is extremely advantageous.

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Croydon Printing Company Ltd

Specification

Title of the Invention

Process for producing 5'-guanylic acid

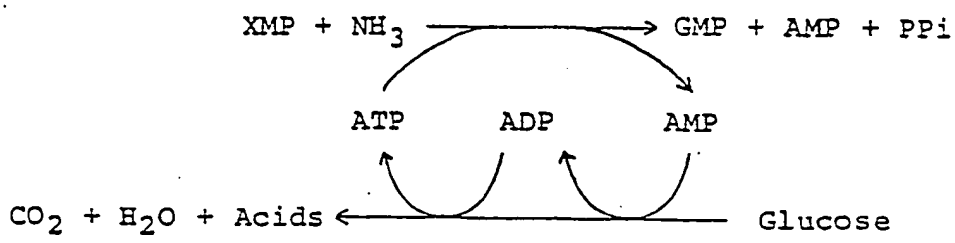
Technical Field

The present invention relates to a process for producing 5'-guanylic acid (hereinafter referred to as GMP). More particularly, it relates to a process for producing GMP by using Escherichia coli having an ability to convert 5'-xanthylic acid (hereinafter referred to as XMP) and ammonia and/or L-glutamine to GMP and also having an ability to convert adenosine monophosphate (hereinafter referred to as AMP) to adenosine triphosphate (hereinafter referred to as ATP) in the presence of an energy donor other than phosphorus oxides, and to a process for producing GMP from XMP by culturing in a medium a transformant obtained by transforming a microorganism by means of a recombinant DNA of a DNA fragment containing a gene of guanylic acid synthetase capable of converting XMP and ammonia and/or L-glutamine to GMP in the presence of ATP (the guanylic acid synthetase is also called xanthylic acid aminase and will be hereinafter referred to as GMP synthetase) and a vector DNA fragment and carrying out reaction using the resulting culture liquor, cells or their treated products as an enzyme source.

Background Art

GMP is in a great demand as a seasoning agent, and the development of a process for producing GMP at lower cost has been desired. Processes for producing GMP so far known include (1) a process by enzymatically decomposing ribonucleic acid, (2) a process by chemically phosphorylating guanosine, (3) a process by converting XMP to GMP by means of bacteria belonging to the genus Brevibacterium or the genus Corynebacterium [Japanese Patent Publication No. 39069/71;

Japanese Patent Application No. 187050/82], etc. Process (3) is an advantageous process using a microorganism capable of reproducing ATP necessary for the conversion from AMP by assimilating cheap energy donors such as glucose, as illustrated below.



Furthermore, a process using a mutant strain whose GMP synthetase activity is intensified by a resistance to chemicals is known [Biotechnol. Bioengineer., 13, 229-240 (1971)].

Disclosure of the Invention

As a result of studies to develop an advantageous process for producing GMP, it has been found that GMP can be produced with Escherichia coli having a GMP synthetase activity and an ability to convert AMP to ATP by utilizing an energy donor other than phosphorus oxides in the presence of phosphate ions and magnesium ions, and that a transformant having a high activity of converting XMP to GMP can be obtained by cloning a gene of GMP synthetase (hereinafter sometimes referred to as guaA) into plasmid vector pBR322, ligating a promoter of tryptophan operon of Escherichia coli (hereinafter referred to as trp promoter or Ptrp) to the upstream side of guaA to construct a recombinant plasmid, and transforming a microorganism with the resulting plasmid.

According to the present invention, XMP and ammonia and/or L-glutamine are converted to GMP in an aqueous medium in the presence of culture or cells of Escherichia coli having a GMP synthetase activity and an ability to convert AMP to ATP

in the presence of an energy donor other than phosphorus oxides (the ability will be hereinafter referred to as ATP reproduction ability) or their treated products and the energy donor other than phosphorus oxides, and GMP is recovered from the reaction solution, whereby GMP can be produced in high yield.

Furthermore, according to the present invention, GMP can be produced in high yield by converting XMP and ammonia and/or L-glutamine to GMP in an aqueous medium in the presence of culture or cells of said transformant belonging to Escherichia coli or their treated products and ATP. When a microorganism having an ATP reproduction ability is used in the process, it is not necessary to add ATP.

Any Escherichia coli (hereinafter sometimes referred to as E. coli) can be used in the present invention so far as it has an ability to form GMP from said substrate. Preferable examples thereof are E. coli PL1068 [see J. Gen. Microbiol., 123, 27-37 (1981)], E. coli KLC421 (guaA$^-$, guaB$^-$) [J. Bact., 139, 320 (1979)], E. coli K294 (r^-, m^+) FERM BP-526, E. coli K294/pXA1 FERM-BP 498, E. coli K294/pXA10 FERM-BP 499, and E. coli K294/pXAR33 FERM BP-500.

The transformant to be used in the present invention can be obtained in the following manner.

The DNA fragment containing a GMP synthetase gene to be used in the present invention includes those derived from procaryotes, bacteriophage and plasmid, among which DNA fragments containing a GMP synthetase gene and derived from Escherichia coli and plasmids containing said DNA fragment are preferred. Specifically, pLC34-10 or pLC32-25, hybrid plasmid of a DNA fragment derived from the chromosome of Escherichia coli and containing both of a gene of inosinic acid dehydrogenase (hereinafter sometimes referred to as guaB) and guaA with DNA of colicin E_1 (hereinafter referred to as ColE_1) is preferable as a guaA source [both are disclosed in Methods in Enzymology 68, 396-408 (1979)], and plasmid DNA is

separated from Escherichia coli JA200 strain containing these plasmids [obtained from Yale University, E. coli Genetic Stock Center (hereinafter referred to as CGSC), disclosed in L. Clarke and J. Carbon, Cell 9, 91-99 (1976)] and purified according to a known method [Nucleic Acids Research 7, 1513 (1979), this method is hereinafter used for the recovery of plasmids].

On the chromosome of Escherichia coli, guaA is positioned just at the downstream side of guaB, and both guaA and guaB genes form one operon together with the promoter operator region at the upstream side of guaB [Molec. gen. Genet. 147, 203-208 (1976)].

When GMP is accumulated in the cells, the expression of both genes is suppressed. Furthermore, it is known that there is a secondary promoter between guaB and guaA (or in the guaB region) which has a lower transcription activity than the promoter at the upstream side [J. Bact. 131, 685-688 (1977)].

Any plasmid can be used for the construction of a recombinant plasmid capable of efficiently expressing the GMP synthetase to be used in the present invention, so far as it can allow the gene of GMP synthetase to be expressed in Escherichia coli. Those having a property of giving such properties as a resistance to antibiotics, etc. to a host microorganism are more preferable. Specific examples thereof include pBR322 [Gene. 2, 95 (1977)] and pBR325 [Gene. 4, 121 (1978)], and more preferably, vectors pGBK3, pGBY1, etc. having a trp promoter and used in Examples of the present invention can be mentioned.

Preparation of the recombinant of the DNA fragment containing a gene and the vector DNA can be carried out according to a known in vitro recombinant DNA technique. The in vitro DNA recombination is usually carried out by physical or enzymatic cleavage and ligation (ligase reaction) of a donor DNA containing a desired gene and a vector DNA. Recovery of the desired recombinant from the ligase reaction

solution can be attained by directly transforming Escherichia coli with the DNA mixture, selectively separating the transformant endowed with a genetic character derived from the genetic information of the desired gene, and isolating the recombinant from the cultured cells of the transformant by extraction. When a vector capable of giving a resistance to chemicals to a host strain such as pBR322, etc. is used, the desired recombinant can also be obtained by first selecting a chemical-resistant strain after the transformation, and then separating the transformant endowed with the genetic character derived from the genetic information of the desired gene.

Any microorganism can be used as a host microorganism according to the present invention, so far as it can express the recombinant DNA and can be utilized to increase the production of GMP, and strains belonging to Escherichia coli and having an ability to incorporate DNA are preferable. Specifically, Escherichia coli PL1068 ($quaA^-$) [see J. Gen. Microbiol. 126, 497-501 (1981)], Escherichia coli K294 (r^-, m^+) FERM BP-526, etc. can be used.

Transformation of a host microorganism with the recombinant DNA can be carried out according to a known method [Cohen et al.: Proc. Natl. Acad. Sci. U.S.A., 69, 2110 (1972), the transformation of E. coli is hereinafter carried out according to this procedure]. Recombinants capable of expressing guaA possessed by the recombinant plasmid in the host cells can be selected by using as a host E. coli PL1068 strains, which are guaA-deficient strains, and selecting the strains which regain the guanine requirement of the host by the transformation.

Culturing of Escherichia coli is carried out in an ordinary medium containing a carbon source, a nitrogen source, inorganic matters, amino acids, vitamins, etc. under aerobic conditions.

As a carbon source, carbohydrates such as glucose, fructose, sucrose, maltose, mannitol, sorbitol, etc., sugar

alcohol, glycerol, starch hydrolyzate solution, molasses, etc. can be used. Furthermore, various organic acids such as pyruvic acid, lactic acid, citric acid, etc., and various amino acids such as glutamic acid, methionine, etc. can be used.

As a nitrogen source, ammonia, various inorganic and organic ammonium salts such as ammonium chloride, ammonium sulfate, ammonium carbonate, ammonium acetate, etc., amino acids such as glutamic acid, glutamine, methionine, etc., or nitrogen-containing organic materials such as peptone, NZ-amine, corn steep liquor, meat extract, yeast extract, casein hydrolyzate, fish meal or its digest, chrysalis hydrolyzate, etc. can be used.

As an inorganic matter, potassium dihydrogen phosphate, sodium monohydrogen phosphate, magnesium sulfate, sodium chloride, calcium chloride, iron chloride, copper sulfate, manganese chloride, ammonium molybdate, zinc sulfate, etc. are added, if necessary. It is not necessary to add vitamines, amino acids, etc. required for the growth of the microorganism to the medium if they are supplied together with other medium components described above.

Culturing is carried out at a temperature of 20 to 50°C, preferably 28 to 42°C and a pH around neutrality under aerobic conditions by shaking culture or aerated stirring culture. The culturing is usually complete in 1 to 24 hours.

The culture of the microorganism can be used as such, and also treated products of the culture, for example, a concentrate or dried product of the culture, a filtrate or cells obtained by centrifuging the culture, dried cells, acetone-treated cells, surfactant-treated cells, organic solvent-treated cells, lytic enzyme-treated cells, immobilized cells, extracted enzyme preparation from the cells, etc. can be used.

Contact reaction can be carried out in any aqueous medium. Most preferably, XMP and ammonia or glutamine, and if

necessary, ATP, an energy donor, phosphate ions, magnesium ions, a surfactant, an organic solvent, etc. are subjected to reaction in the culture liquor of a microorganism, whereby GMP is accumulated in the culture liquor, or the said components are added to the culture liquor, cells or their treated products after the completion of culturing, and subjected to reaction at 20 to 50°C for 1 to 48 hours, whereby GMP can be accumulated. In that case, it is desirable to adjust the pH to 6-10. As an ammonium source, an ammonium salt is usually used. Concentrations (g/ℓ) of the substrates and additives in the medium or reaction solution are 1-100 XMP, 1-100 ATP, 1-50 $MgSO_4 \cdot 7H_2O$, 1-25 $(NH_4)_2SO_4 \cdot 7H_2O$, and 1-25 glutamine.

As an XMP source, any of those containing XMP, such as a microbial XMP fermentation liquor as such, a concentrate thereof, its partially purified preparation, etc. can be used besides the highly purified preparation, so long as it does not inhibit the formation of GMP.

As an energy donor, any of carbohydrates such as glucose, arabinose, lactose, maltose, sucrose, mannitol, sorbitol, trehalose, molasses, starch hydrolyzate, etc.; organic acids such as pyruvic acid, lactic acid, acetic acid, α-ketoglutaric acid, etc.; amino acids such as glycine, alanine, aspartic acid, glutamic acid, etc. can be used, so far as it is a non-phosphatized compound and can be utilized by the Escherichia coli used. These are used at a concentration of 1-200 g/ℓ.

It is desirable to keep the concentration of phosphate ions and magnesium ions in a range of 4-400 mM in the contact reaction solution. When the amount of the ions taken into the raction solution from the culture liquor or cells falls within the said range of concentration, it is not necessary to add the ions thereto, whereas when the ions are insufficient, they are added so that the amount will fall within the said range of concentration. As phosphate ions, any of sodium salt, potassium salt, magnesium salt, etc. of

phosphoric acid can be used. As magnesium ions, any of inorganic salts and organic acid salts can be used.

As a surfactant, cationic surfactants such as polyoxyethylene stearylamine (e.g. Nimin S-215, made by Nippon Oil and Fats Co., Ltd.), cetyl-trimethylammonium bromide, etc.; anionic surfactants such as sodium oleylamide sulfate, etc., non-ionic surfactants such as polyoxyethylene sorbitan monostearate (e.g. Nonion ST221, made by Nippon Oil and Fats Co., Ltd.), etc., amphoteric surfactants such as laurylbetaine (e.g. Anon BF, made by Nippon Oil and Fats Co., Ltd.), etc. can be used. They are usually used at a concentration of 0.1 to 50 g/ℓ, preferably 1 to 20 g/ℓ.

As an organic solvent, toluene, xylene, analiphatic alcohol, benzene, ethyl acetate, etc. can be used at a concentration of 0.1 to 50 mℓ/ℓ, preferably 1 to 20 mℓ/ℓ.

GMP accumulated in the aqueous reaction solution can be recovered according to the ordinary procedure using activated carbon, ion exchange resin, etc.

Brief Description of the Drawings

Fig. 1 shows a process for constructing plasmid pXAR33.
Fig. 2 shows a process for constructing plasmid pGC7.
Fig. 3 shows a process for constructing plasmid pGKA2.
Fig. 4 shows a process for constructing plasmid pGBK3.
Fig. 5 shows a process for constructing plasmid pGBY1.

Best Mode for Carrying Out the Invention

Example 1

Construction of a recombinant plasmid which efficiently expresses GMP synthetase

1) Subcloning of guaA into pBR322:

E. coli JA200 strain carrying pLC34-10, hybrid plasmid of gua operon (containing guaA and guaB) derived from the chromosome of *E. coli* and ColE$_1$ was inoculated in L medium

containing 10 g/ℓ Bacto-Tryptone (made by Difco Co.), 5 g/ℓ yeast extract (made by Difco Co.) and 5 g/ℓ sodium chloride and adjusted to pH 7.2, and cultured at 30°C for 18 hours. Plasmid pLC34-10 was separated from the cultured cells and purified according to the known procedure described above. pBR322 used as a vector was separated from E. coli JA194 strain which carries it [B. Ratzlein and J. Carbon, Proc. Natl. Acad. Sci. U.S.A. 74, 487 (1977)] and purified in the same manner as above. For the culturing and preservation of microorganisms described hereinafter, L medium was used unless otherwise specified.

pLC34-10, which has a size of about 15 kilobases (which will be hereinafter abbreviated as Kb), was cleaved with restriction enzyme EcoRI at one position and with PstI at three positions (see Fig. 1). It was expected that there was guaA on the EcoRI-PstI fragment of about 7 kb containing one PstI cleavage site [Biochem. Biophys. Res. Commun., 72, 1129-1136 (1976)].

Then, 5µg of pLC34-10 plasmid DNA prepared above was dissolved in 50 µℓ of a buffer solution containing 10 mM Tris-hydrochloric acid (pH 7.5), 50 mM NaCl, 7 mM $MgCl_2$ and 6 mM 2-mercaptoethanol (hereinafter referred to as "Y-50 buffer solution"), and 20 units of restriction enzyme EcoRl [made by Takara Shuzo Co., the restriction enzymes hereinafter are all products of Takara Shuzo Co. unless otherwise specified] was added thereto. The mixture was subjected to digestion reaction at 37°C for 2 hours, and then 5 units of PstI (made by New England Biolabs Co.) was added thereto. The mixture was subjected to partial digestion reaction at 37°C for 30 minutes, and the reaction was discontinued by heat treatment at 65°C for 10 minutes. A plasmid DNA fragment of about 7 Kb was purified from the digest by low-gelling-temperature agarose gel electrophoresis [Analytical Biochemistry, 98, 305 (1979)] (this procedure was hereinafter used for the purification of DNA fragments). Separately, 2 µg of pBR322

plasmid DNA was dissolved in 20 µℓ of Y-50 buffer solution, and 8 units of EcoRI and 8 units of PstI were added thereto. The mixture was subjected to digestion reaction at 37°C for 2 hours, and the larger plasmid fragment (about 3.6 Kb) was purified.

Then, about 0.2 µg of the DNA fragment derived from pLC34-10 and about 0.05 µg of the DNA fragment derived from pBR322 which were obtained above were treated with 2 units of T4 ligase at 4°C for 18 hours in 40 µℓ of a buffer solution containing 20 mM Tris-hydrochloric acid (pH 7.6), 10 mM $MgCℓ_2$, 10 mM dithiothreitol and 0.5 mM ATP (hereinafter referred to as "T4 DNA ligase buffer solution"). E. coli PL1068 strain mutated at guaA was transformed with the thus obtained recombinant plasmid DNA according to the procedure of Cohen, et al. described above, whereby a transformant having a resistance to tetracycline (20 µg/mℓ) and being devoid of the guanine requirement shown by the host was obtained.

A plasmid was separated and purified from the transformant, and structural analysis of the plasmid was made by digesting the DNA with restriction enzymes such as EcoRI, PstI, etc. As a result, it was found to be a recombinant plasmid wherein the EcoRI-PstI DNA fragment (about 7 Kb) derived from pLC34-10 was inserted at the EcoRI-PstI site of pBR322, and named pXA1. E. coli KLC421 ($guaA^-$, $guaB^-$) [J. Bact. 139, 320 (1979)] transformed with pXA1 was smeared onto M9 plate medium (containing 1g of $NH_4Cℓ$, 6g of Na_2HPO_4, 3g of KH_2PO_4, 5g of NaCl, 0.25g of $MgSo_4 \cdot 7H_2O$, 3g of glucose, 4g of vitamin B_1, and 2g of Casamino acid in 1ℓ of water, and also 1.5% agar) to investigate the growth. It was found that it grew on the plate medium containing 5 mg/ℓ xanthine or guanine, whereas no growth was observed on the M9 plate medium containing 5 mg/ℓ hydroxanthine. This result shows that pXA1 carries only guaA derived from pLC34-10 though pLC34-10 carries both guaA and guaB. The strain obtained by transforming E. coli K294 with pXA1 was deposited with the

Fermentation Research Institute, Agency of Industrial Science and Technology as E. coli K294/pXA1 FERM BP-498 on March 8, 1984.

2) Ligation of trp promoter (hereinafter referred to as Ptrp) to the upstream side of guaA:

First, 3 µg of pXA1 DNA was dissolved in 30 µℓ of Y-50 buffer solution, and 15 units of HindIII was added thereto. The mixture was subjected to digestion reaction at 37°C for 2 hours, and 2 µℓ of 2M NaCℓ and 15 units of MluI were added thereto. The mixture was subjected to digestion reaction at 37°C for 2 hours. After heat treatment at 65°C for 10 minutes, the smaller DNA fragment containing guaA (about 3.3 Kb) was purified. On the other hand, pGBK3 was used as a plasmid containing Ptrp. A procedure for constructing pGBK3 is shown in Reference Example 2. pGBK3 has a HindIII cleavage site at the downstream side of Ptrp (see Fig. 1).

3 µg of pGBK3 DNA was digested with HindIII and MluI in the same manner as above, and the larger DNA fragment containing Ptrp (about 4 Kb) was purified. Then, about 0.2 µg of the DNA fragment derived from pXA1 and about 0.1 µg of the DNA fragment derived from pGBK3 which were obtained above were subjected to ligation reaction at 4°C for 18 hours in 20 µℓ of T4DNA ligase buffer solution in the presence of one unit of T4DNA ligase. E. coli K294 strain was transformed with the thus obtained recombinant plasmid, whereby a transformant having a resistance to ampicillin was obtained. A plasmid DNA was separated and purified from the transformant, and by structural analysis, it was confirmed that the plasmid had the structure wherein the DNA fragment containing guaA and derived from pXA1 was inserted at the downstream side of Ptrp derived from pGBK. The plasmid was named pXA10 (see Fig. 1). E. coli strain carrying pXA10 was deposited with the Fermentation Research Institute as E. coli K294/pXA10 FERM BP-499 on March 8, 1984.

3) Shortening of the distance between Ptrp and guaA:

As described later, no substantial difference was recognized in the expression of guaA between the strain carrying pXA10 and the strain carrying pXA1 (see Table 1).

Thus, plasmid pXAR33 having a shortened distance between Ptrp and guaA was constructed in the following manner (see Fig. 1).

First, about 10 µg of pXA10 DNA was dissolved in 60 µℓ of a buffer solution containing 10 mM Tris-hydrochloric acid (pH 7.5), 150 mM NaCℓ, 7 mM $MgCℓ_2$ and 2-mercaptoethanol (hereinafter referred to as "Y-150 buffer solution"), and 40 units of HindIII was added thereto. The mixture was subjected to digestion reaction at 37°C for 2 hours. To 30 µℓ of the HindIII-digested reaction solution were added 20 µℓ of Bal31 (made by Bethesda Research Laboratories), and the mixture was subjected to digestion reaction at 30°C. During the period of 5 to 50 minutes after the start of reaction, the reaction solution was sampled in portions of 10 µℓ from time to time, and each portion was put into 20 µℓ of phenol : chloroform mixture (1:1 by volume). The mixture was thoroughly stirred to discontinue the reaction, and was ice cooled. After centrifuging, an upper layer was sampled, and 2-fold volume of ice-cooled ethanol was added thereto. The mixture was allowed to stand at -80°C for 30 minutes. After the respective ethanol mixtures were centrifuged, the supernatant was discarded, while 50 µℓ of Y-150 buffer solution at a 10-fold concentration and 43 µℓ of distilled water were added to the precipitates to dissolve the precipitates. Then, 3 units of MluI was added to the respective solutions, and the solutions were subjected to digestion reaction at 37°C for 2 hours. The reaction solutions were heat-treated at 65°C for 10 minutes, and the smaller DNA fragments (not more than 3 Kb) were purified. On the other hand, plasmid pGBY1 in which the NruI site on the DNA fragment derived from pBR322 of pGBK3 was changed to BglII site was used as a vector (the procedure for constructing pGBY1 is shown in Reference Example 3).

3 µg of pGBY1 DNA was dissolved in 20 µℓ of Y-50 buffer solution, and 15 units of HindIII was added thereto. The mixture was subjected to digestion reaction at 37°C for 2 hours. After the reaction, the reaction solution was subjected to extraction with phenol and chloroform, and to precipitation with ethanol. The DNA fragment was dissolved in 40 µℓ (total volume) of 50 mM Tris-hydrochloric acid (pH 7.6), 7 mM $MgCℓ_2$, 6 mM 2-mercaptoethanol, 0.25 mM dATP, 0.25 mM dGTP, 0.25 mM dTTP and 0.25 mM dCTP, and then 6 units of E. coli DNA polymerase I·Klenow fragment (1 µℓ, made by New England Biolabs) was added thereto. The mixture was subjected to reaction at 15°C for 2 hours, and the 5'-protruding end formed by HindIII digestion was changed to a blunt end.

After the extraction with phenol-chloroform and the precipitation with ethanol, the precipitate of DNA was dissolved in 20 µℓ of Y-150 buffer solution, and 15 units of MluI was added thereto. The mixture was subjected to digestion reaction at 37°C for 2 hours. The larger DNA fragment having Ptrp (about 4.2 Kb) was purified from the MluI-digestion reaction product by low-gelling-temperature agarose gel electrophoresis.

To about 0.1 µg each of the thus obtained DNA fragments derived from pXA10 was added 0.05 µg of the DNA fragment derived from pGBY1. The respective mixtures were subjected to ligation reaction at 4°C for 18 hours in 20 µℓ (total volume) of T4 DNA ligase buffer solution in the presence of one unit of T4 DNA ligase. Then, E. coli K294 strains were transformed with the thus obtained recombinant plasmid DNA, and the transformants having a resistance to ampicillin were selected. The obtained ampicillin-resistant strains were cultured in M9 liquid medium (M9 plate medium freed from agar) at 30°C for 18 hours, and the GMP synthetase activity was investigated in the manner described below to select strains having a higher activity than that of E. coli K294 strain carrying pXA10. Plasmid DNAs were separated from

the strains having a higher activity (R-33 strain) and purified, and the structures thereof were analyzed. It was found that plasmid pXAR33 possessed by the R-33 strain had the HindIII-MluI DNA fragment containing guaA of pXA10. Among the analyzed plasmid DNAs, pXAR33 had the shortest DNA fragment, and the R-33 strain which carried it showed a nigh GMP synthetase activity. The R-33 strain was deposited with the Fermentation Research Institute as Escherichia coli K294/pXAR33 FERM BP-500 on March 8, 1984.

4) GMP synthetase activity of the strains carrying recombinant plasmids:

Determination of GMP synthetase activity was carried out according to a known procedure [J. Biol. Chem., 226, 351-363 (1957)] after modification as described below.

Seed culture of E. coli for the activity determination test was inoculated in M9 liquid medium and subjected to shaking culture at 30°C for 18 hours. The culture liquor was diluted with distilled water or concentrated by suspending in an appropriate amount of distilled water after centrifugation, and then toluene was added thereto to make a final concentration of 20 mℓ/ℓ. The mixture was shaken at 37°C for 20 minutes.

The toluene-treated culture liquor was put into a reacting solution comprising 160 mM Tris-hydrochloric acid (pH 8.6), 12 mM ATP, 25 mM XMP, 16 mM $MgSO_4 \cdot 7H_2O$ and 40 mM $(NH_4)_2SO_4$, and the mixture was shaken at 42°C to conduct conversion of XMP to GMP.

GMP formed was quantitatively determined by sampling the reaction solution from time to time, mixing the sample with 40-fold volume of 3.5% perchloric acid, centrifuging the mixture, and measuring the absorbance of the supernatant at 290 nm.

In Table 1, the GMP synthetase activities of the strains used or obtained in the present invention are shown,

wherein one unit of activity is defined as an amount of activity which forms 1 μmole of GMP per minute.

Table 1

Host E. coli strain	Plasmid	Deposit No.	Activity per wet cell (unit/g wet cell)
K294	-	FERM BP-526	0.88
K294	pXA1	FERM BP-498	17.08
K294	pXA10	FERM BP-499	16.91
K294	pXAR33	FERM BP-500	73.50

Example 2

The culture liquor (wet cell weight: 4.6 mg/mℓ) of pXAR33-carrying strain (R-33) obtained in Example 1 was concentrated 40-fold by centrifugation, and toluene was added thereto to make a final concentration of 20 mℓ/ℓ. The mixture was shaken at 37°C for 20 minutes. Then, 30 mℓ of a reaction solution comprising 20 mg/mℓ XMP·Na_2·$7H_2O$, 20 mg/mℓ ATP·Na_2·$3H_2O$ and 10 mg/mℓ $(NH_4)_2SO_4$ and adjusted to pH 8.6 was placed in a 200 mℓ-beaker, and 0.2 mℓ of said cell suspension containing toluene was added thereto. The reaction mixture was stirred by means of a magnetic stirrer (900 rpm), and kept at 42°C for 5 hours, while adjusting the pH to 8.6 with caustic soda. As a result of the reaction, 13.0 mg/mℓ 5'-GMP·Na_2·$7H_2O$ was formed in the reaction solution. When the host K294 strain was used in place of the R-33 strain, the yield was less than 1 mg/mℓ.

Example 3

R-33 strain was inoculated in a 300 mℓ-Erlenmeyer flask containing 30 mℓ of L medium and subjected to shaking culture at 30°C for 18 hours by means of a rotary shaker (220 rpm). To the culture liquor were added toluene, XMP·Na_2·$7H_2O$, ATP·Na_2·$3H_2O$ and $(NH_4)_2SO_4$ to make 20 μℓ/mℓ,

20 mg/mℓ, 20 mg/mℓ and 10 mg/mℓ, respectively. Shaking culture was continued for additional 10 hours while keeping the temperature at 42°C and adjusting the pH to 8.6 with caustic soda. As a result, 13.7 mg/mℓ GMP·Na_2·$7H_2O$ was formed and accumulated in the medium. When the host K294 strain was used in place of the R-33 strain, the yield was less than 1 mg/mℓ.

Example 4

E. coli K294 (FERM BP-526) strain was inoculated in a 1ℓ-Erlenmeyer flask containing 200 mℓ of M9 medium comprising 6 g/ℓ disodium phosphate, 3 g/ℓ potassium dihydrogen phosphate, 5 g/ℓ sodium chloride, 1 g/ℓ ammonium chloride, 4 mg/ℓ thiamine hydrochloride, 250 mg/ℓ magnesium sulfate and 3 g/ℓ glucose, and subjected to reciprocating shaking culture at 28°C overnight. The cells were collected by centrifugation, and preserved in a frozen state (-20°C).

Water was added to the frozen cells at room temperature to make a suspension at final concentration of 200 g/ℓ as wet cell weight, and 40 g/ℓ XMP·Na_2·$7H_2O$, 50 g/ℓ glucose, 2 g/ℓ sodium phytate, 5 g/ℓ Na_2HPO_4 and 5 g/ℓ $MgSO_4 \cdot 7H_2O$ were dissolved in the suspension (concentrations are final ones). The resulting mixture was put into 200 mℓ-beakers in portions of 20 mℓ. The respective beakers were kept at 37°C in a thermostat water bath and stirred at 900 rpm by means of magnetic stirrers for 24 hours to convert XMP to GMP, while adjusting the pH to 7.4 with 9% aqueous ammonia (see Table 2). In Table 1, (1) shows the result obtained by using the reaction mixture of said composition as such and (2) shows the result obtained by using the reaction mixture of said composition further admixed with 4 g/ℓ Nimin S-215 (Nimin is hereinafter abbreviated as NIM) and with 10 mℓ/ℓ xylene.

Table 2

	NIM S-215 and xylene	$GMP \cdot Na_2 \cdot 7H_2O$ (g/ℓ)
(1)	-	2.2
(2)	+	5.2

Example 5

E. coli K294 strain and *E. coli* K294/pXA10 strain were cultured in the same manner as in Example 4 except that M9 medium containing 50 mg/ℓ ampicillin was used for the culturing of K294/pXA10 strain. As a result of reaction under the same conditions as in Example 4-(2) using NIM S-215 and xylene, the *E. coli* K294 strain formed 5.5 g/ℓ $GMP \cdot Na_2 \cdot 7H_2O$ in 23 hours, and the *E. coli* K294/pXA10 strain formed 23.8 g/ℓ $GMP \cdot Na_2 \cdot 7H_2O$ in 6 hours.

Example 6

E. coli K294/pXA10 strain was cultured in M9 medium containing 50 mg/ℓ ampicillin in the same manner as in Example 4. The amount of cells was 7.5 g/ℓ as wet cell weight. To the culture liquor were added XMP, glucose, sodium phytate, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, NIM S-215 and xylene to make 40 g/ℓ, 50 g/ℓ, 2 g/ℓ, 5 g/ℓ, 5 g/ℓ, 4 g/ℓ and 10 mℓ/ℓ, respectively, and conversion of XMP to GMP was conducted in the same manner as in Example 4. As a result, 5.2 g/ℓ $GMP \cdot Na_2 \cdot 7H_2O$ was formed in 23 hours.

Reference Example 1

Construction of recombinant plasmid pGKA2 which expresses human interferon (IFN)-γ (see Figs. 2 and 3):

(a) Insertion of human IFN-γ DNA into the expression vector pKYP11:

In this example, 6 µg of plasmid pIFNγ-G4 which was isolated from ATCC 39123 strain by the above-described method for isolation of a plasmid was dissolved in 50 µℓ (total volume) of a solution containing 20 mM Tris-hydrochloric acid (Tris-HCℓ) (pH 7.5), 10 mM $MgCℓ_2$, 10 mM dithiothreitol and 50 mM NaCℓ. Then, 12 units each of restriction enzymes PvuII and HindIII were added and digestion reaction was carried out at 37°C for 4 hours. The reaction solution was heated at 65°C for 7 minutes to inactivate the enzymes and subjected to purification by low-gelling-temperature agarose gel electrophoresis to obtain 1.2 µg of a DNA fragment containing human IFN-γ DNA of 1.3 Kb.

Separately, 4 µg of pKYP11 was dissolved in 40 µℓ (total volume) of a solution containing 20 mM Tris-HCℓ (pH 7.5), 10 mM $MgCℓ_2$, 10 mM dithiothreitol and 50 mM NaCℓ. 3 units of BamHI was added and digestion reaction was carried out at 37°C for 3 hours. The reaction solution was heated at 65°C for 5 minutes to inactivate the enzyme. Thereafter, 30 µM each of dATP, dCTP, dGTP and dTTP were added and 8 units of Escherichia coli DNA polymerase I (Klenow fragment, product of New England Biolabs, 1 µℓ) was added. Fill-in reaction was carried out at 15°C for 1 hour and the reaction solution was heated at 68°C for 15 minutes to inactivate the DNA polymerase I. 10 units of HindIII was added and digestion reaction was carried out at 37°C for 3 hours, followed by heating at 65°C for 5 minutes to inactivate the HindIII.

The digestion reaction solution of the plasmid pKYP-11 thus obtained was subjected to purification by low-gelling-temperature agarose gel electrophoresis to obtain about 2.5 µg of a DNA fragment of about 4.7 Kb containing Ptrp.

Then, 0.5 µg of the DNA fragment of 1.3 Kb containing human IFN-γ DNA and 1.0 µg of the DNA fragment of about 4.7 Kb containing Ptrp, which was obtained from the plasmid pKYP11, were dissolved in 20 µℓ of a solution containing 20 mM Tris-HCℓ (pH 7.5), 6 mM $MgCℓ_2$, 5 mM

dithiothreitol and 500 μM ATP, and 4 units of T4 DNA ligase (product of New England Biolabs) was added. Ligation reaction was carried out at 4°C for 18 hours, and Escherichia coli HB101 was transformed with the obtained recombinant plasmid mixture by conventional technique to obtain an Ap^R colony. A plasmid, pGC7 illustrated in Fig. 2 was separated from the culture broth of the colony. The structure of pGC7 was confirmed by digestion with HindIII, BamHI, HpaI, SaI, EcoRI and ClaI and agarose gel electrophoresis. Escherichia coli strain carrying pGC7 has been deposited with the Fermentation Research Institute as Escherichia coli IGC7 (FERM BP-497).

(b) Construction of recombinant plasmid pGKA2:

In this example, 6 μg of pGC7 DNA obtained in Reference Example 1(a) was dissolved in 50 μℓ (total volume) of a solution containing 20 mM Tris-HCℓ (pH 7.5), 10 mM $MgCℓ_2$, 10 mM dithiothreitol and 10 mM NaCℓ, and 12 units of restriction enzyme BstNI (product of New England Biolabs) was added. Reaction was carried out at 60°C for 3 hours. Then, 150 mM NaCℓ and 8 units of SalI were added and digestion reaction was carried out at 37°C for 3 hours. The reaction solution was again heated at 65°C for 5 minutes to inactivate the SalI and subjected to purification by low-gelling-temperature agarose gel electrophoresis to obtain about 0.8 μg of a DNA fragment of about 1,125 bp containing a large portion of the human IFN-γ DNA.

Separately, 3 μg of pKYP10 was dissolved in 40 μℓ (total volume) of a solution containing 20 mM Tris-HCℓ (pH 7.5), 10 mM $MgCℓ_2$, 10 mM dithiothreitol and 100 mM NaCℓ. 6 units each of restriction enzymes HindIII and SalI were added and digestion reaction was carried out at 37°C for 3 hours. The reaction solution was heated at 65°C for 5 minutes to inactivate HindIII and SalI and subjected to purification by low-gelling-temperature agarose gel electrophoresis to obtain about 1.8 μg of a DNA fragment of about 4.1 Kb containing Ptrp.

The N-terminal amino acid of the mature human IFN-γ polypeptide is Cys. In order to express mature IFN-γ DNA, it is necessary to furnish an initiation codon (ATG) just before the 5'-terminal codon TGT (Cys) and further to adjust the length between SD-sequence at the downstream side of Ptrp and ATG to a suitable length of 6-18 bp. Therefore, the following DNA linker was synthesized.

```
HindIII                  Met Cys Tyr Cys  BstNI
5' AGCTT ATG TGTTACTGCC 3'        (18-mer)
      3' ATACACAATGACGGT 5'        (15-mer)
```

Two single chain DNAs of 18-mer and 15-mer were synthesized by a conventional tri-ester method [R. Crea, et al.: Proc. Natl. Acad. Sci., 75, 5765 (1978)]. Then, 2 µg each of the 18-mer and 15-mer DNAs were dissolved in 20 µℓ (total volume) of a solution containing 50 mM Tris-HCℓ (pH 7.5), 10 mM $MgC\ell_2$, 5 mM dithiothreitol, 0.1 mM EDTA and 1 mM ATP. 30 units of T4 polynucleotide kinase (product of Boehringer Mannheim) was added and phosphorylation reaction was carried out at 37°C for 60 minutes.

Then, 2 µg each of phosphorylated 18-mer and 15-mer DNAs were mixed and the mixture was heated at 70°C for 5 minutes and allowed to stand at room temperature for annealing to obtain the DNA linker having the structure given above.

0.4 µg of the BstNI - SalI fragment of 1,125 bp obtained above and derived from pGC7 and 1.0 µg of the DNA fragment of 4.1 Kb obtained by digestion of the expression vector pKYP10 with HindIII and SalI were dissolved in 25 µℓ (total volume) of a solution containing 20 mM Tris-HCℓ (pH 7.5), 6 mM $MgC\ell_2$, 5 mM dithiothreitol and 500 µM ATP. About 0.1 µg of the DNA linker mentioned above was added to the mixture, followed by addition of 6 units of T4 DNA ligase. Ligation reaction was carried out at 4°C for 17 hours. Escherichia coli HB101 was transformed using the obtained

recombinant plasmid mixture by conventional technique to obtain an Ap^R colony. A plasmid, pGKA2 illustrated in Fig. 3 was isolated from the culture broth of the colony. The structure of pGKA2 was confirmed by digestion with EcoRI, ClaI, HindIII, BstNI and SalI and agarose gel electrophoresis. It was confirmed by the method of Maxam-Gilbert [A.M. Maxam, et al.: Proc. Natl. Acad. Sci., 74, 560 (1977)] that the base sequence from the SD-sequence (AAGG) to the initiation codon (ATG) in the plasmid pGKA2 was "AAGGGTATCGATAAGCTTATG".

The human IFN-γ DNA in pGKA2 is different from known DNAs in that the DNA has RsaI site and the ninth amino acid of the human IFN-γ polypeptide encoded by the DNA is glutamine (Gln).

Further, the synthesized DNA used above is different from the one used by P.W. Gray, et al. having the following structure:

```
                    m e t c y s t y r c y s
AATTC ATG TGTTATTGTC
      GTACACAATAACAGT
```

in the underlined parts. Thus, pGKA2 has a restriction site for BstNI, CCAGG, in the DNA region coding for human IFN-γ and this feature also distinguishes pGKA2 from known plasmids. Furthermore, the length and structure between the SD-sequence and ATG are important because of influence on the expression of proteins in Escherichia coli. The base sequence between the SD-sequence and ATG in pGKA2 is apparently different from that in the known recombinant plasmid pIFN-γ trp48 (P.W. Gray, et al.)

Escherichia coli carrying pGKA2 has been deposited with the Fermentation Research Institute as Escherichia coli IGKA2 (FERM BP-496).

Reference Example 2

Construction of recombinant plasmid pGBK3 which expresses human IFN-γ under control of tacI promoter:

As the first step for constructing the recombinant plasmid, insertion of the transcription termination site of Escherichia coli lipoprotein (lpp) gene (hereinafter referred to as lpp terminator) into IFN-γ-expressing plasmid pGKA2 (the procedure for constructing pGKA2 is shown in Reference Example 1) was carried out according to the following procedures (a), (b), (c) and (d) (see Fig. 4).

(a) Construction of pGBD1:

In this example, 2 µg of plasmid pIFNγ-G4 (about 3.6 Kb) was dissolved in 20 µℓ of Y-50 buffer solution, and 6 units of PvuII was added thereto. Digestion reaction was carried out at 37°C for 2 hours, and then discontinued by heat treatment at 65°C for 10 minutes. Then, 0.1 µg of the digest was subjected to ligation reaction at 4°C for 18 hours with 2 units of T4 DNA ligase in 20 µℓ of T4 DNA ligase buffer solution in the presence of 5 picomoles of 5'-phosphorylated BamHI linker (5'-pCCGGATCCGG-3'; made by Collaborative Research Co.).

Escherichia coli HB101 strain was transformed with the thus obtained recombinant plasmid DNA to obtain an Ap-resistant colony. A plasmid DNA was isolated from the transformant, and the DNA was digested with restriction enzymes such as BamHI, etc. to conduct structural analysis of the plasmid. As a result, it was confirmed that recombinant plasmid pGBD1 wherein BamHI linker was inserted at the PvuII site of pIFNγ-G4 was obtained.

(b) Construction of pKYP14:

Construction of recombinant plasmid pKYP14 used as a source of the lpp terminator is described below.

First, 5 µg of trp promoter-carrying plasmid pKYP10 (Japanese Unexamined Published Patent Application No. 110600/83) was dissolved in 40 µℓ of Y-100 buffer solution, and 10 units of BamHI was added thereto. Digestion reaction was carried out at 37°C for 2 hours, and then 1 µℓ of Y-100 buffer solution, 2.5 µℓ of 1M NaCℓ, 5.5 µℓ of distilled water and 20 units of SaℓI were added thereto. The reaction was further carried out at 37°C for 2 hours. After heat treatment at 65°C for 10 minutes, the larger plasmid DNA fragment (about 4.9 Kb) was purified by low-gelling-temperature agrarose gel electrophoresis. Separately, 5 µg of lpp terminator-carrying plasmid pIN-II-A1 [K. Nakamura, et al.: The EMBO Journal 1, 771 (1982)] (the same as pKEN045 in Japanese Unexamined Published Patent Application No. 140800/82) was digested with BamHI and SaℓI in the same manner as above. The resulting BamHI-SaℓI fragment of about 0.95 Kb containing the lpp terminator was purified.

Then, about 0.1 µg of the DNA fragment derived from pKYP10 and about 0.05 µg of the DNA fragment derived from pIN-II-A1 which were obtained above were subjected to ligation reaction at 4°C for 18 hours in 20 µℓ of T4 DNA ligase buffer solution in the presence of one unit of T4 DNA ligase.

A plasmid DNA was isolated from the Escherichia coli HB101 strain transformed with the thus obtained recombinant plasmid DNA, and subjected to structural analysis, whereby it was confirmed that the lpp terminator was inserted at the downstream side of plasmid pKYP14 possessed by the IKYP14 strain.

(c) Construction of pGBJ2:

Insertion of lpp terminator at the downstream side of IFN-γDNA was carried out by recombining recombinant plasmids pGBD1 and pKYP14 obtained in the foregoing procedures (a) and (b) in the following manner.

First, 5 µg of plasmid pGBD1 (about 3.6 Kb) was dissolved in 30 µℓ of a buffer solution containing 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$ and 6 mM 2-mercaptoethanol (hereinafter referred to as "Y-0 buffer solution"), and 10 units of ClaI was added thereto. Digestion reaction was carried out at 37°C for two hours. After heat treatment at 65°C for 10 minutes and subsequent ice cooling, 2 µℓ of Y-0 buffer solution at 10-fold concentration, 5 µℓ of 1M NaCℓ, 12 µℓ of distilled water and 2.0 units of restriction enzyme BamHI were added thereto and mixed. Digestion reaction was carried out at 37°C for 2 hours. The plasmid DNA was partially digested with BamHI during the reaction. The resulting ClaI-BamHI DNA fragment (about 1.3 Kb) containing IFN-γ DNA was purified. Separately, 5 µg of plasmid pKYP14 (about 5.8 Kb) containing lpp terminator was digested with 10 units of ClaI and 20 units of BamHI in 50 µℓ of Y-50 buffer solution for 2 hours, and then the larger plasmid DNA fragment of about 5.0 Kb containing the lpp terminator was purified. The thus obtained DNA fragment derived from pKYP14 (about 0.1 µg) and DNA fragment derived from pGBD1 (about 0.05 µg) were subjected to ligation reaction at 4°C for 18 hours in 20 µℓ of T4 DNA ligase buffer solution in the presence of one unit of T4 DNA ligase.

A plasmid DNA was isolated from the Escherichia coli HB101 strain transformed with the thus obtained recombinant plasmid, and subjected to structural analysis, whereby it was confirmed that plasmid pGBJ2 possessed by the IGBJ2 strain had the structure wherein the lpp terminator was inserted at the downstream side of IFN-γDNA.

(d) Construction of pGBK3:

Plasmid pGBK3 having the structure wherein the lpp terminator is inserted at the downstream side of IFN-γ DNA was constructed by recombining recombinant plasmid pGBJ2 obtained in the foregoing step (c) and IFN-γ-expressing plasmid pGKA2 (see Reference Example 1) in the following manner.

First, about 5 µg of plasmid pGKA2 (about 5.2 Kb) was dissolved in 30 µℓ of Y-50 buffer solution, and more than 10 units of PstI was added thereto. Digestion reaction was carried out at 37°C for 2 hours. After heat treatment at 65°C for 10 minutes and subsequent ice cooling, 2 µℓ of Y-150 buffer solution at 10-fold concentration, 3 µℓ of 1M NaCl, 14 µℓ of distilled water and 10 units of restriction enzyme NcoI (made by New England Biolabs, the same restriction enzyme was used hereinafter) were added thereto, and digestion reaction was carried out at 37°C for 2 hours. After heat treatment at 65°C for 10 minutes, a plasmid DNA fragment of about 1.85 Kb containing IFN-γ DNA was purified. Then, about 5 µg of recombinant plasmid pGBJ2 (about 6.4 Kb) obtained above was subjected to the same treatment as applied to pGKA2, and the resulting PstI-NcoI plasmid DNA fragment of about 4.7 Kb was purified. The thus obtained DNA fragment derived from pGKA2 (about 0.1 µg) and DNA fragment derived from pGBJ2 (about 0.1 µg) were subjected to ligation reaction at 4°C for 18 hours in 20 µℓ of T4 DNA ligase buffer solution in the presence of one unit of T4 DNA ligase. A plasmid DNA was separated and purified from the Escherichia coli HB101 strain transformed with the thus obtained recombinant plasmid, and subjected to structural analysis, whereby it was confirmed that plasmid pGBK3 possessed by the IGBK3 strain had the desired structure.

Reference Example 3

Construction of pGBY1 (see Fig. 5):

Recombinant plasmid pGBY1 wherein BglII linker is inserted at the NruI site of recombinant plasmid pGBK3 obtained in Reference Example 2 (d) was obtained in the following manner.

First, about 2 µg of plasmid pGBK3 (about 5.9 Kb) was dissolved in 20 µℓ of Y-100 buffer solution, and 4 units of NruI (made by New England Biolabs) was added thereto.

Digestion reaction was carried out at 37°C for 2 hours, and discontinued by heat treatment at 65°C for 10 minutes. Then, 0.1 µg of the digest was subjected to ligation reaction at 4°C for 18 hours with 2 units of T4 DNA ligase in 20 µl of T4 DNA ligase buffer solution in the presence of 5 pM of 5'-phosphorylated BglII linker (5' pCAGATCTG-3'; made by Takara Shuzo Co.).

Escherichia coli HB101 strain was transformed with the thus obtained recombinant plasmid DNA to obtain an Ap-resistant colony. A plasmid DNA was isolated from the tranformant, and the structural analysis of the plasmid was carried out by digesting the DNA with restriction enzymes such as BglII, etc., whereby it was confirmed that recombinant plasmid pGBY1 wherein BglII linker was inserted at the NruI site of pGBK3 was obtained.

Claims

1. A process for producing 5'-guanylic acid (GMP), characterized by converting 5'-xanthylic acid (XMP), and ammonia and/or L-glutamine to GMP in an aqueous solution in the presence of culture or cells of Escherichia coli having an ability to convert XMP and ammonia and/or L-glutamine to GMP in the presence of adenosine triphosphate (ATP) and also an ability to convert adenosine monophosphate to ATP in the presence of an energy donor other than phosphorous oxides or their treated products and the energy donor other than phosphorus oxides, and recovering GMP from the reaction solution.

2. A process according to Claim 1, wherein the Escherichia coli is a strain obtained by transforming Escherichia coli with a recombinant DNA of a DNA fragment containing a gene of guanylic acid synthetase and a vector DNA.

3. A process according to Claim 1, wherein the conversion reaction is carried out in the course of culturing of the Escherichia coli.

4. A process according to Claim 1, wherein the conversion reaction is carried out in the presence of at leas one member selected from phosphate ions, magnesium ions, a surfactant and an organic solvent.

5. A process according to Claim 1, wherein the conversion reaction is carried out in the presence of at leas one energy donor selected from carbohydrates, organic acids, and amino acids.

6. A process for producing GMP, characterized by converting XMP and ammonia and/or L-glutamine to GMP in an aqueous medium in the presence of culture or cells of a strain obtained by transforming a microorganism with a recombinant DNA of a DNA fragment containing a gene of guanylic acid synthetase and a vector fragment, or their treated products and ATP, and recovering GMP from the reaction solution.

7. A process according to Claim 6, wherein the vector DNA contains a tryptophan promoter.

8. A process according to Claim 6, wherein the microorganism is Escherichia coli.

9. A process according to Claim 6, wherein the conversion reaction is carried out in the course of culturing of the microorganism.

10. A process according to claim 6, wherein the conversion reaction is carried out in the presence of at least one member selected from phosphate ions, magnesium ions, a surfactant and an organic solvent.

11. Escherichia coli K294/pXA1, FERM BP-498.

12. Escherichia coli K294/pXA10, FERM BP-499.

13. Escherichia coli K294/pXAR33, FERM BP-500.

Fig. 1

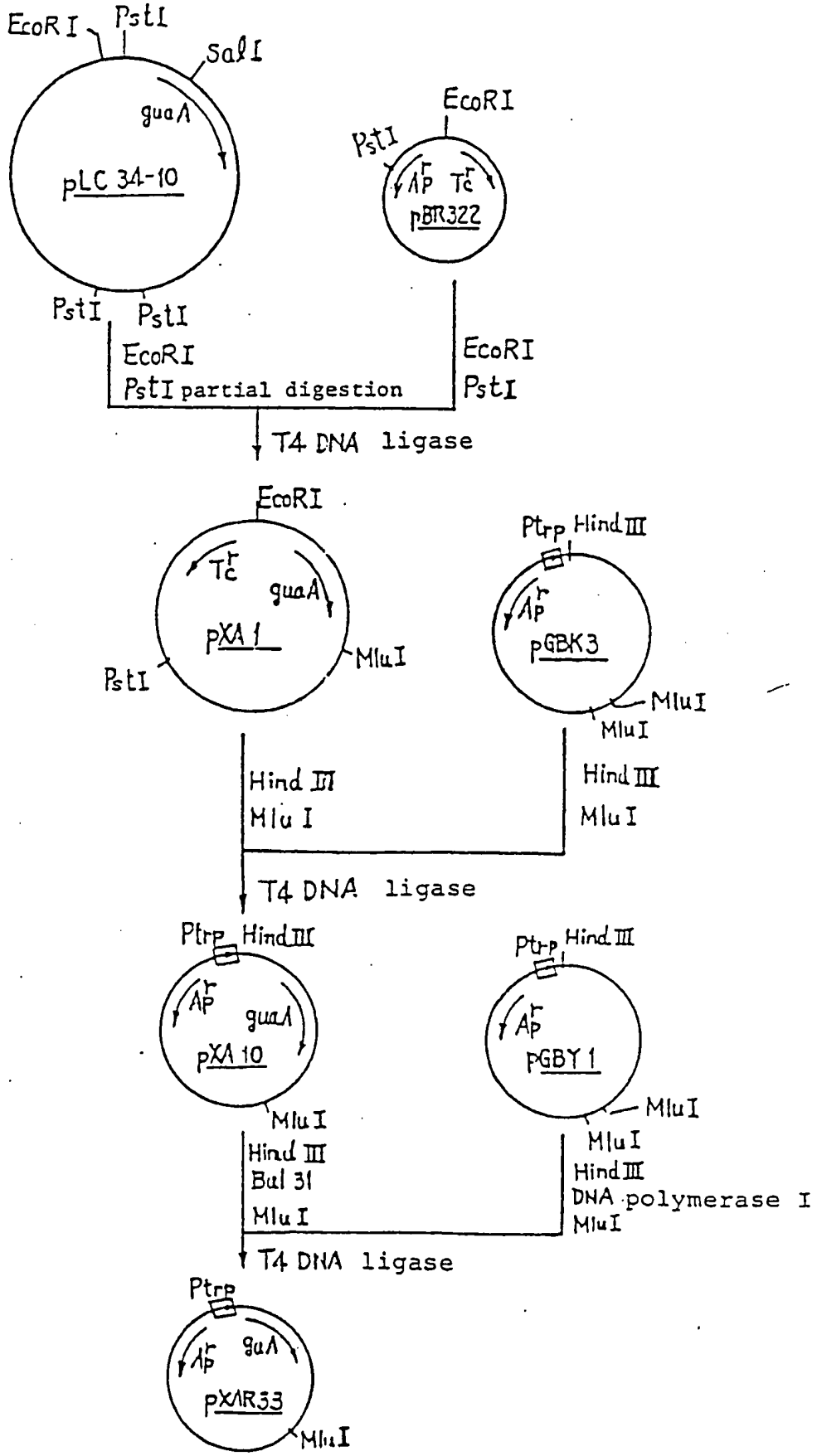


Fig. 2

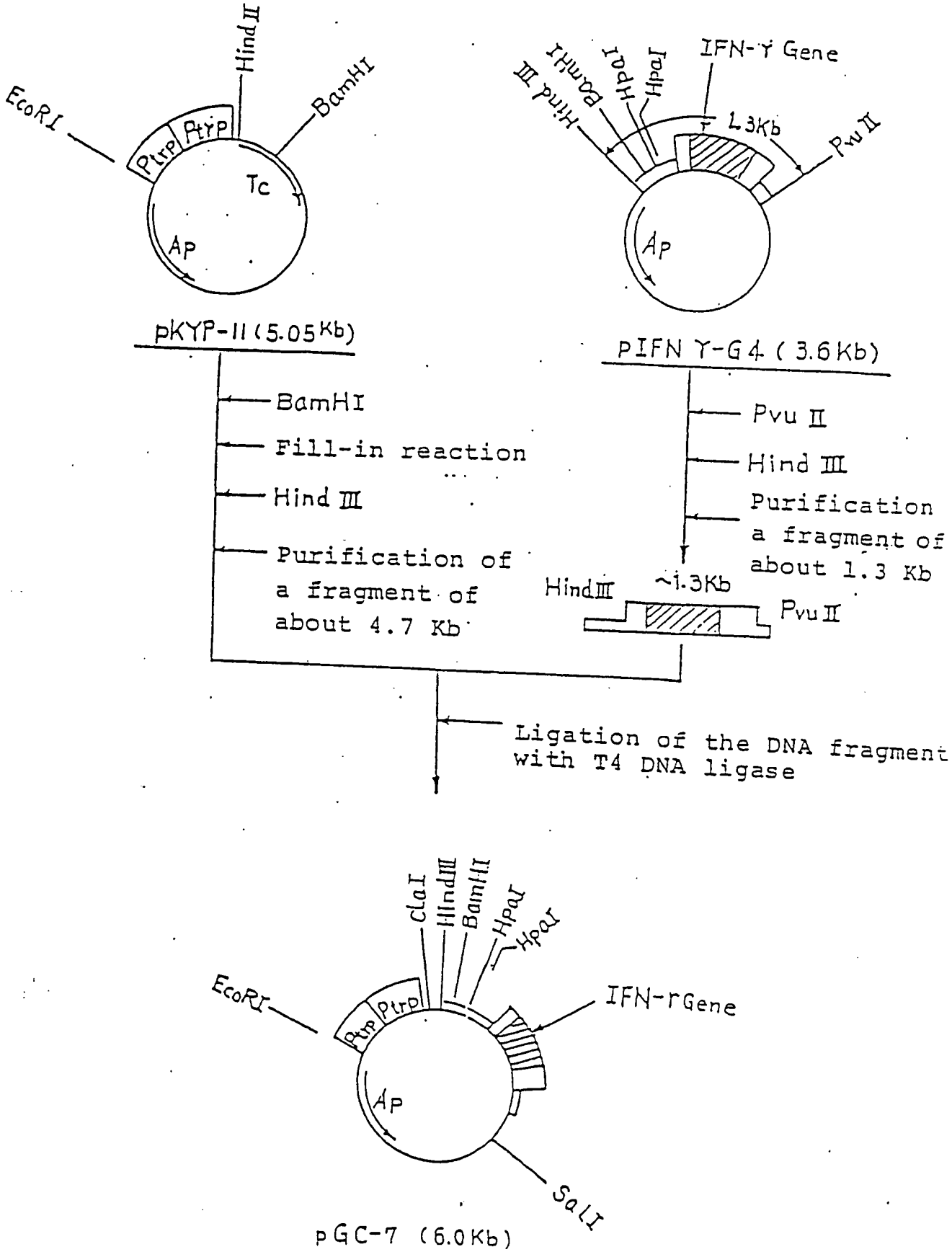


Fig. 3

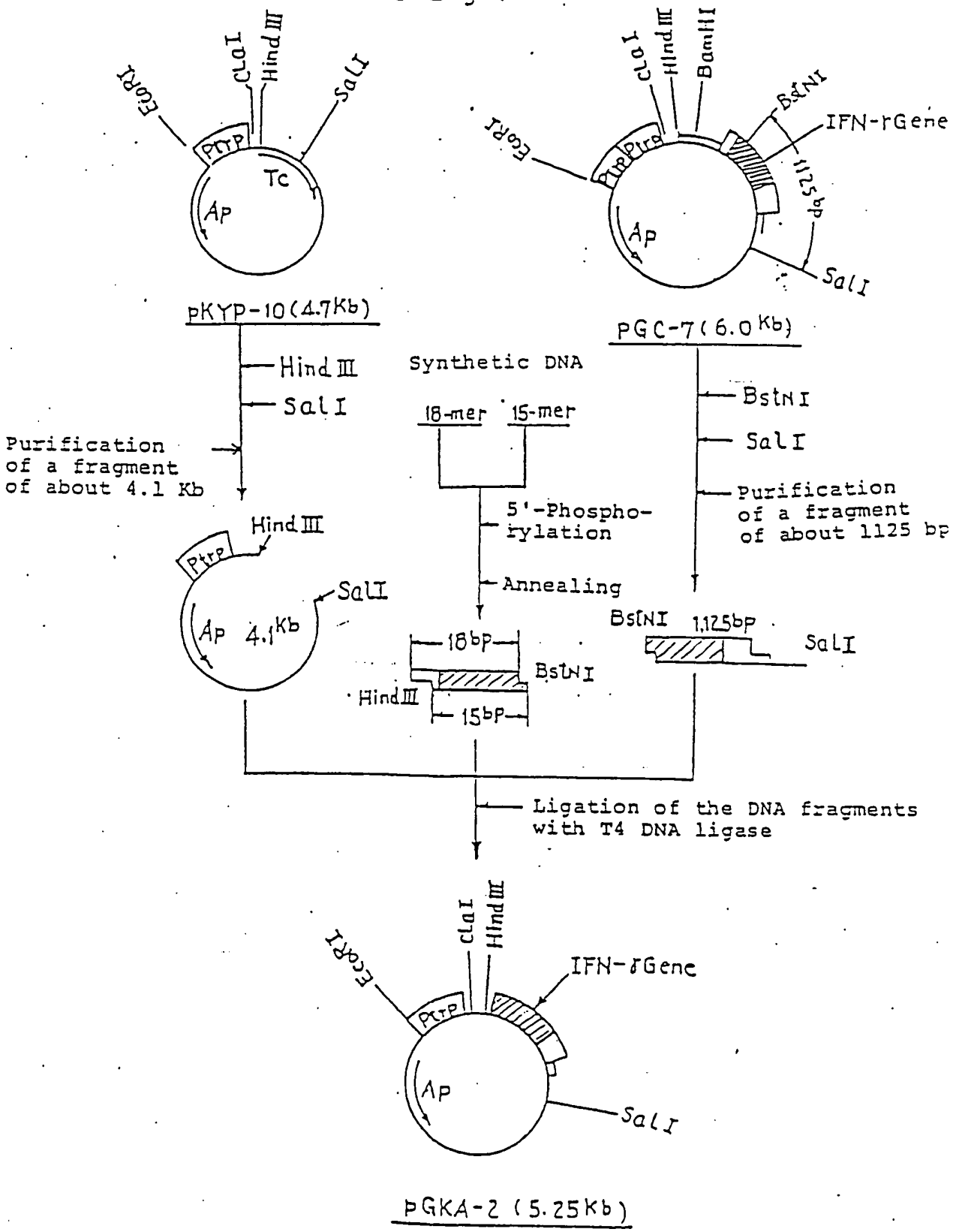


Fig. 4

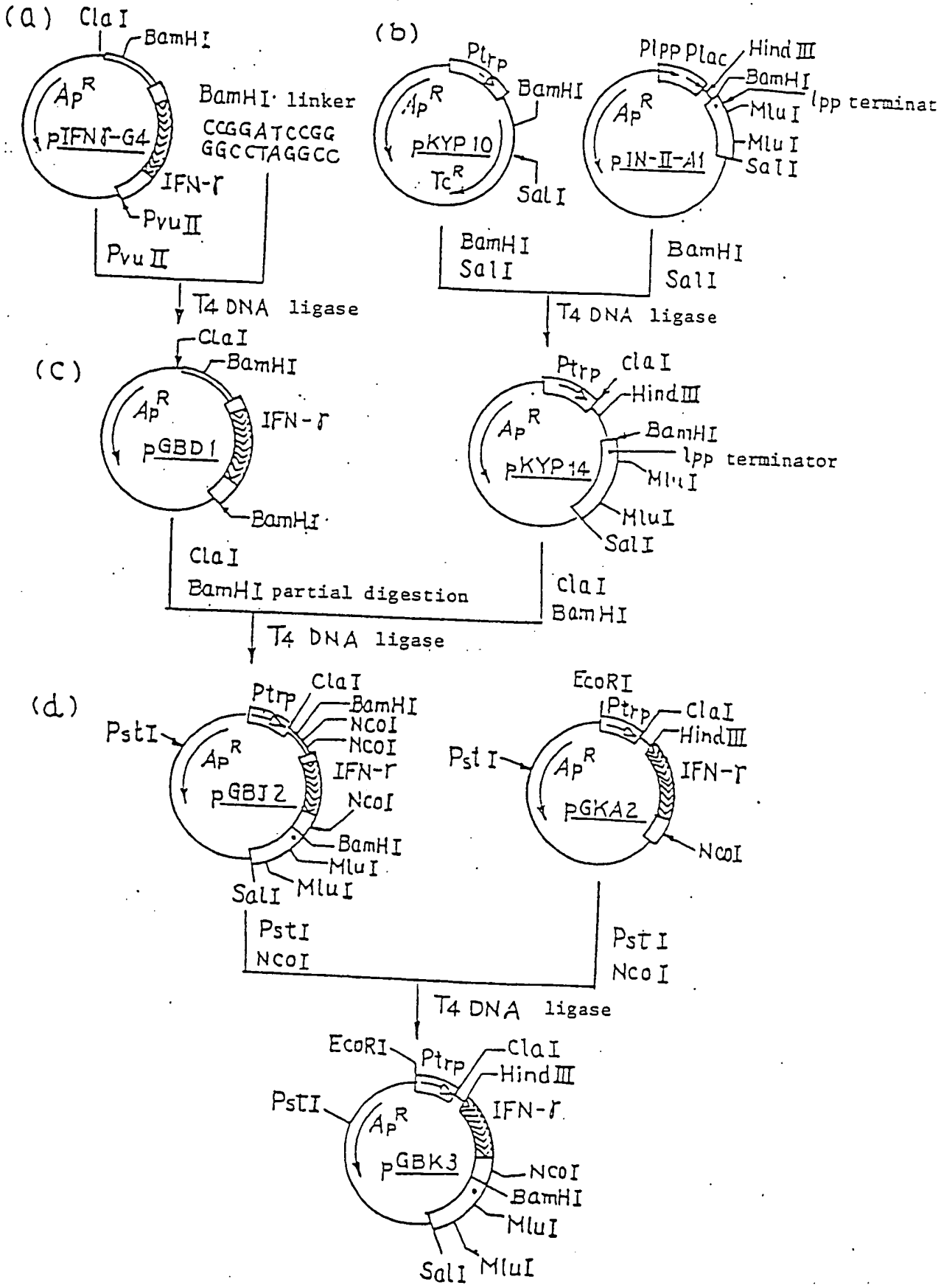
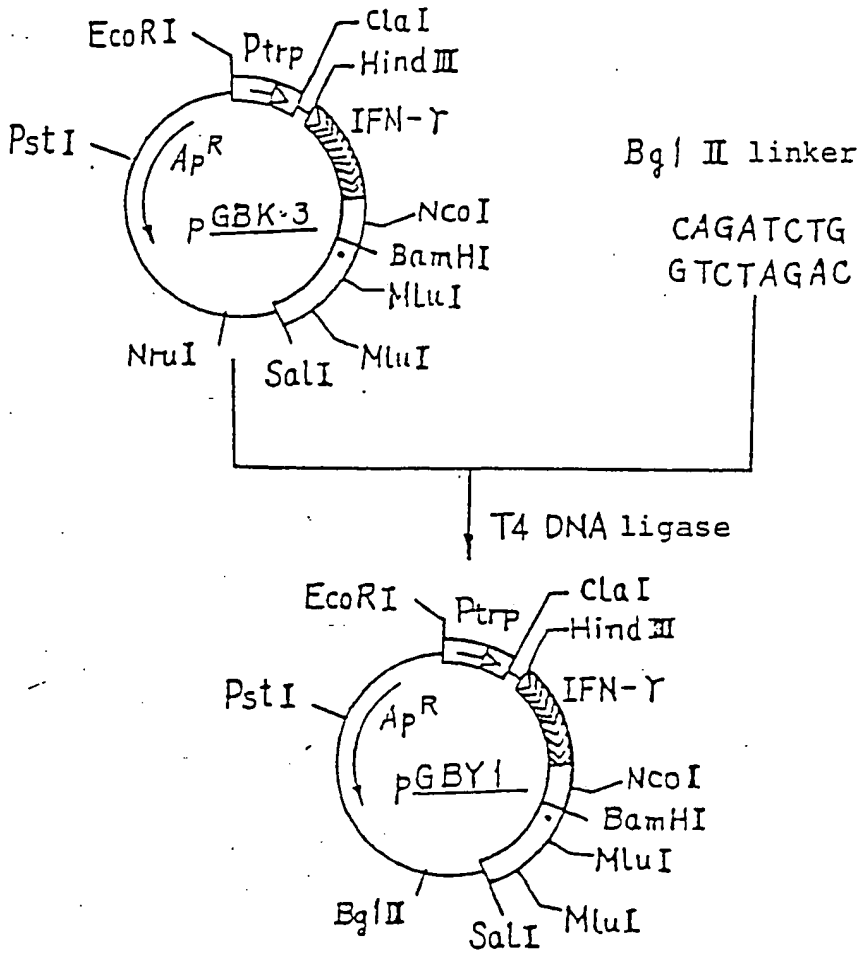


Fig. 5



INTERNATIONAL SEARCH REPORT

0185092

International Application No. PCT/JP85/00122

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl[4] C12P 19/32, C12N 15/00 // (C12P 19/32, C12R 1:19)

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC	C12P 19/32, C12N 15/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category*	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	JP, B2, 57-166992 (Seitetsu Kagaku Kogyo Kabushiki Kaisha) 14 October 1982 (14. 10. 82) (Family : none)	6
A	JP, B2, 58-111697 (Ajinomoto Co., Inc.) 2 July 1983 (02. 07. 83) (Family : none)	1, 6
A	JP, B2, 58-175492 (Ajinomoto Co., Ltd.) 14 October 1983 (14. 10. 83) (Family none)	6

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [1]	Date of Mailing of this International Search Report [1]
May 30, 1985 (30. 05. 85)	June 3, 1985 (03. 06. 85)
International Searching Authority [1]	Signature of Authorized Officer [20]
Japanese Patent Office	

Form PCT/ISA/210 (second sheet) (October 1981)